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**ACETYLCHOLINESTERASE ACTIVITY IN
SYNAPTIC VESICLES OF THE MEDULLA
OF RATS EXPOSED TO HIGH DOSES
OF GAMMA-NEUTRON RADIATION**

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ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE
Defense Atomic Support Agency
Bethesda, Maryland

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ACETYLCHOLINESTERASE ACTIVITY IN SYNAPTIC VESICLES
OF THE MEDULLA OF RATS EXPOSED TO HIGH DOSES OF
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FOREWORD
(Nontechnical summary)

Synaptic vesicles are small sac-shaped organelles containing the neurotransmitting agent acetylcholine. Localized along the external membrane of the synaptic vesicle is acetylcholinesterase, a hydrolytic enzyme.

During a synapse, an impulse is transmitted across the synaptic gap. This impulse is initiated by acetylcholine. The primary means for termination of impulses is by the inactivation of acetylcholine by acetylcholinesterase. Therefore, acetylcholinesterase (ACHE) acts as a controlling mechanism for impulse transmission. Indeed, the absence of ACHE or the reduction of normal ACHE activity could be catastrophic, resulting in a neurosynaptic block (uncontrolled or blocked transmissions of impulses). This condition, which can be potentiated by drugs such as eserine (physostigmine), can be functionally incapacitating resulting in numerous physiological problems leading to death.

The present study was therefore designed to determine the effect radiation has on ACHE activity at doses considered to be incapacitating. It was observed that the activity or concentration of the enzyme was significantly reduced after irradiation. This possibly could have been due to increased permeability of the synaptic membrane along with a reduction in functional vesicles due to a complete rupture or destruction of the vesicle membrane. In addition, the damage is believed to occur shortly after irradiation.

ABSTRACT

Sprague-Dawley rats were subjected to a whole-body dose of 15 krad of mixed gamma-neutron radiation. At 2 and 24 hours following irradiation 14 animals from the irradiated group and 14 unirradiated controls were sacrificed by replacing the blood in the brain circulation by perfusion with a sucrose- eserine solution. The medulla section of the brain was removed, homogenized and prepared for ultracentrifugation and sucrose density gradient separation. The separated synaptic vesicles were assayed for protein and acetylcholinesterase (ACHE). A decrease in protein was found 2 hours after irradiation with a greater decrease 22 hours later. A decrease in ACHE activity was also seen 2 hours after irradiation but there was no further decrease at 24 hours. These results were correlated with vascular changes found during the central nervous system (CNS) syndrome.

I. INTRODUCTION

Acetylcholinesterase, an enzyme found along the external membrane of synaptic vesicles, has as its role hydrolysis of acetylcholine.^{5,9,10} By doing so it ends a previous impulse transmission and allows the start of subsequent impulses. Under normal conditions the balance of the transmitter substance, acetylcholine (ACH), and the hydrolyzing agent, acetylcholinesterase (ACHE), is such that impulses are under complete control during nerve stimulation. It has been reported that radiation causes a reduction in ACHE activity and an increase in ACH activity in various areas of the brain.^{1,4,11,12,14,15} This could indicate functional or structural changes in the synaptic vesicles. Also, animals injured by relatively high doses of radiation (greater than 2 krads) experience prior to death a set of symptoms termed the central nervous system (CNS) syndrome. During the early stages of the CNS syndrome the animals experience changes in blood pressure and respiration.² These physiological functions are primarily controlled by areas located in the medulla. It is then possible to postulate that changes in the concentrations of ACH or ACHE induced by high doses of radiation might cause alterations of the physiological functions of these brain areas. Therefore, the object of the present experiment was to measure possible postirradiation changes in the concentration of ACHE in synaptic vesicles of areas of the medulla oblongata which control blood pressure and to correlate them with functional deterioration.

II. MATERIALS AND METHODS

Fifty-six Sprague-Dawley rats weighing approximately 200 grams were divided into two groups. One group was exposed to 15 krads of whole-body mixed gamma-neutron

radiation from the AFRRI-TRIGA reactor, while the other group served as an unirradiated control.

At 2 hours and at 24 hours following irradiation, 14 animals from each group (control and experimental) were anesthetized with 0.25 ml of Sodium Nembutal (50 mg/ml) and the blood in the brain circulation replaced by perfusion with a sucrose-eserine solution (0.32 M and 5×10^{-5} M respectively). Sucrose (0.32 M) was used alone with no difference in results. The brains from the animals were removed and the medulla along with a portion of the brainstem was dissected and separated. This portion of the brain of each group of animals was weighed and placed in 20 ml of cold sucrose (1:10 w/v) where it was minced and then homogenized.

The homogenate was centrifuged at 1000 g for 10 minutes to remove red blood cells and nuclear debris. The supernatant was centrifuged twice for 20 minutes at 12,000 g. The resultant pellet termed P_2 was resuspended in 2.5 ml of the solution used for homogenization and was saved for ultracentrifugation and sucrose density gradient separation. The remaining supernatant was centrifuged again for 60 minutes at 30,000 g and the new supernatant was discarded. The new pellet termed P_3 was resuspended in 2.5 ml of sucrose solution and was also saved for ultracentrifugation. The P_2 and P_3 pellets resuspended in sucrose solution were layered over a continuous sucrose density gradient, density range 1.12 - 1.19, with an 8-ml layer of heavy sucrose on the bottom to act as a cushion. They were then centrifuged for 3-1/2 hours at 90,000 g. Two-milliliter fractions were collected and the synaptic membrane was broken by freezing and thawing 10 times. In order to locate the fractions containing acetylcholinesterase in the density gradient separation, the amount of protein in each

fraction was determined by the method of Lowry et al.⁸ The fractions were subsequently analyzed for acetylcholinesterase (ACHE). ACHE activity was determined by the method of Ellman et al.⁶ with acetylthiocholine iodide as the substrate buffered with 0.1 M phosphate buffer, pH 8.0. The color reagent was buffered dithiobisnitrobenzoic acid (DTNB). Portions of the synaptic vesicle fractions were checked for purity with the Siemens Elmiskop 1A electron microscope for both P₂ and P₃ fractions.

III. RESULTS

The results of the sucrose density gradient show the P₂ fraction to be separated into three distinct bands (Figure 1). The uppermost band consisted of soluble protein and clumps of floating synaptic vesicles found between densities of 1.12 - 1.14. This was followed by the microsomal band at densities of 1.15 - 1.16 and the mitochondrial band found between densities of 1.17 - 1.19.

The results of the P₃ fractions were very similar with one exception, the absence of a mitochondrial band, which was eliminated during preparative centrifugation.

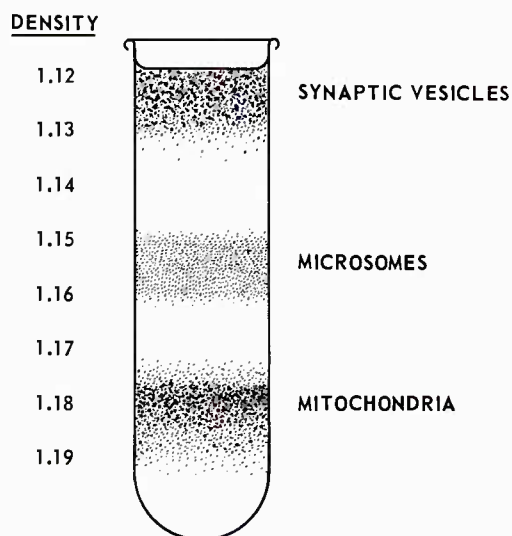


Figure 1. Continuous sucrose density gradient separation of the P₂ fraction

Figures 2 and 3 illustrate the results of the Lowry protein assay on the collected 2-ml samples of both the control and irradiated P_2 and P_3 fractions. All the samples were assayed for acetylcholinesterase (ACHE). Those samples indicating the highest specific activity for ACHE were found to contain primarily synaptic vesicles. These were fractions 24 and 25 on Lowry protein assays (Figures 2 and 3).

Figure 2 illustrates the results of the P_2 fractions of control animals and animals 2 hours and 24 hours postirradiation. The results as seen in fractions 24 and 25 indicate a significant decrease ($p < .01$) in the protein concentration of samples containing synaptic vesicles in irradiated rats when compared with those of the unirradiated controls.

The 2-hour 24th and 25th fractions (Figure 2) show an average decrease of 12.6 percent in protein from control values ($p < .01$) while the decrease in the 24-hour sample is 42.8 percent ($p < .01$). When the irradiated fractions are compared with each other, the 24-hour sample demonstrates a 34.6 percent decrease in protein ($p < .01$).

A similar assay of the P_3 fraction for 24 and 25 (Figure 3) shows no significant changes in protein concentration between the irradiated samples and the unirradiated controls.

The results of the ACHE assay for the synaptic vesicle samples for both P_2 and P_3 pellets are presented in Table I. In the samples collected from the control animals, the rate (R) of hydrolysis is 4.02 and 3.24 $\mu\text{moles}/\text{min per g}$ for P_2 and P_3 respectively, where R is the rate in μmoles substrate hydrolyzed per minute per gram of tissue and depends on substrate concentration. Similar measurements in the 2-hour postirradiated animals resulted in R equal to 1.31 for P_2 and 1.20 for P_3

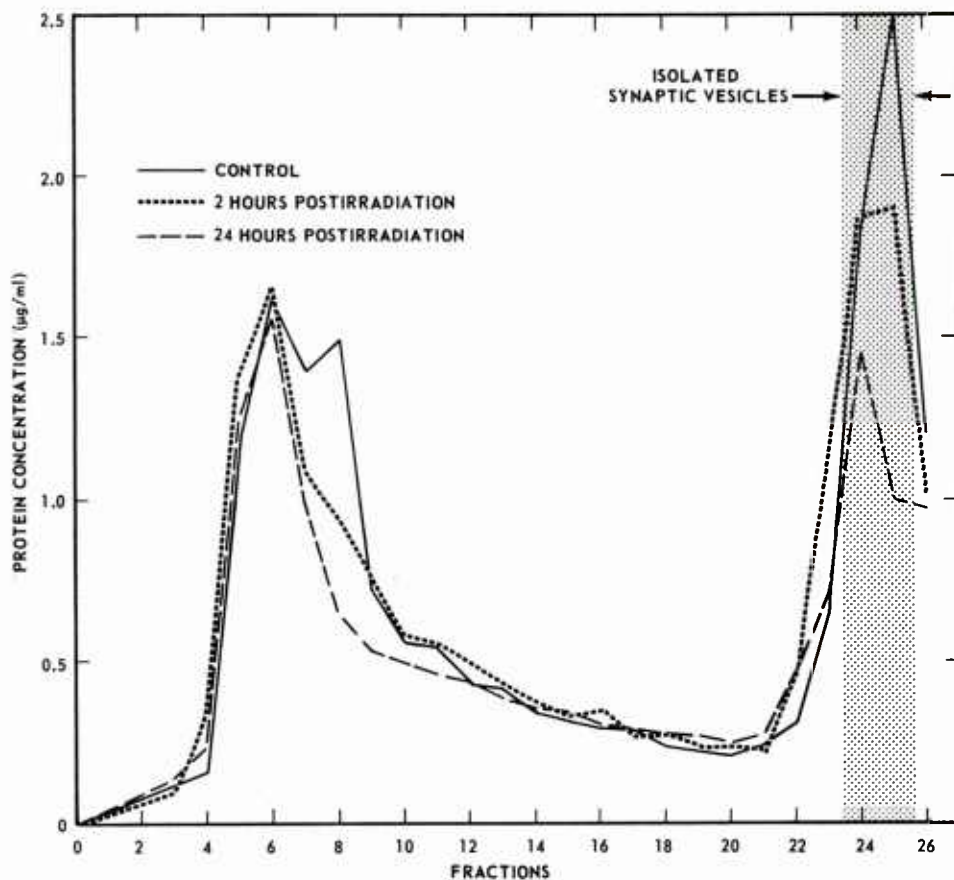


Figure 2. Lowry protein assay of the P_2 fractions collected from a sucrose density gradient tube following high speed centrifugation of medulla homogenate (90,000 g for 3-1/2 hours) obtained from animals exposed to 15 krad of mixed gamma-neutron radiation and sacrificed 2 and 24 hours after exposure and from unirradiated controls.

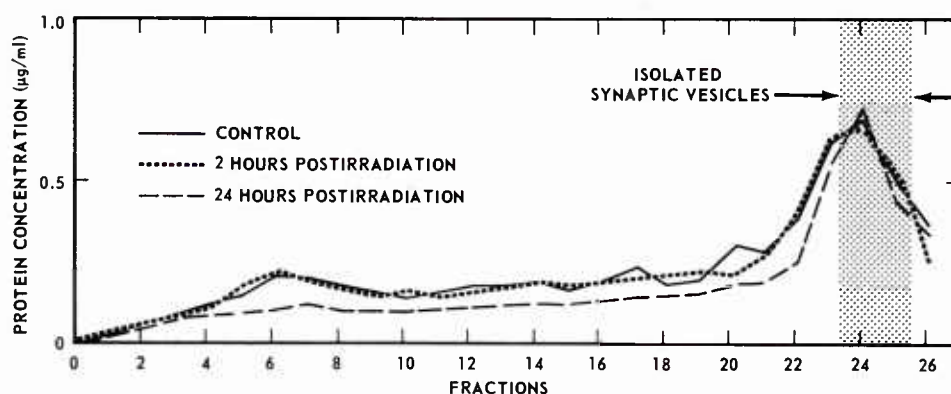


Figure 3. Lowry protein assay of the P_3 fractions collected from a sucrose density gradient tube following high speed centrifugation of medulla homogenate (90,000 g for 3-1/2 hours) obtained from animals exposed to 15 krad of mixed gamma-neutron radiation and sacrificed 2 and 24 hours after exposure and from unirradiated controls.

Table I. Enzyme Activity (ACHE) in Samples of Synaptic Vesicles of Medulla Homogenates of Control and Irradiated Rats (15 krad, sacrificed 2 and 24 hours after exposure)

	P_2^*		P_3^*	
	Rate†	Percent decrease	Rate†	Percent decrease
Control	4.02		3.24	
2 hours	1.31‡	67	1.20‡	62
24 hours	1.28‡	68	1.16‡	62

* P_2 and P_3 represent two stages of purity of the synaptic vesicles in the separation procedure

† μ moles/min per g

‡ p value is $< .01$

which is significantly different ($p < .01$) from the controls. The 24-hour postirradiation samples were also significantly different from the control values ($P_2 = 1.28$ and $P_3 = 1.16$). There were no significant differences between the 2-hour and 24-hour postirradiation samples.

IV. DISCUSSION

This study clearly shows a decrease in ACHE activity in both the P_2 and P_3 fractions of synaptic vesicles 2 and 24 hours after exposure. These data also suggest that the damage causing the decrease in ACHE activity occurs within 2 hours because the ACHE activity does not show any further decrease 24 hours after exposure. This decrease in activity might be caused by increased permeability of the synaptic vesicle membrane permitting release of ACH. The resulting increase in free ACH outside of the synaptic vesicles would then be hydrolyzed by the ACHE found along the synaptic vesicle membrane. This could possibly account for the observed net decrease in ACHE. However, even if ACH release continues at the normal or even an increased

rate and is hydrolyzed at a reduced rate due to radiation-induced reduction in AChE synthesis, ACh concentration might be increased. Indeed, reports by several investigators using various animals and radiation doses have shown an increase in ACh postirradiation.^{1,11,12,14,15} In addition, due to the isolation procedure employed in this study, we were able to isolate almost pure synaptic vesicles which gives us a better model of the ACh-AChE synaptic complex without influence from other brain systems.

In light of the experimental conditions utilized, the present data suggest that at supralethal doses, there is probably complete alteration of the synaptic vesicle membrane to the point of total destruction, resulting in a drastic drop in AChE activity. This is attested to by a decrease in protein concentration in collected synaptic vesicles 2 hours after irradiation with a greater decrease 24 hours postirradiation. There is also ample evidence that supralethal doses of radiation cause damage to vessels and capillaries of brain tissue and the blood-brain barrier.² This is indicated by an increase in capillary permeability accompanied by hemorrhage and edema.² Collectively these symptoms are part of the total central nervous system (CNS) syndrome. In addition, radiation doses as low as 25 R have been found to increase the sensitivity of the blood-brain barrier to ACh in mice.⁷ There are also results which show that administration of AChE inhibitors to animals increases the permeability of the blood-brain barrier.¹⁵ It is then possible to postulate that the AChE decrease and/or ACh increase after high doses of irradiation might play a considerable role in the CNS syndrome. Also, since our results indicate early AChE reduction within 2 hours, the possibility arises that the ACh-AChE synaptic complex might be responsible in

some way for the early temporary incapacitation (ETI) reported by Seigneur and Brennan¹³ in monkeys and Casarett and Comar³ in rats.

The present studies have indicated a decrease in the activity of ACHE in the synaptic vesicles of the irradiated rats' medulla. This decrease has been attributed to increased permeability of the synaptic vesicle membrane possibly leading to its complete destruction. The present data also suggest that the decreased ACHE activity accompanied by increased ACH activity^{1, 11, 12, 14, 15} might play a considerable role in the alteration of permeability in the blood-brain barrier and/or the vascular system. Finally, it is suggested that these physiological disturbances are involved in producing early incapacitation in animals subjected to high doses of ionizing radiation.

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13. ABSTRACT <p>Sprague-Dawley rats were subjected to a whole-body dose of 15 krad of mixed gamma-neutron radiation. At 2 and 24 hours following irradiation 14 animals from the irradiated group and 14 unirradiated controls were sacrificed by replacing the blood in the brain circulation by perfusion with a sucrose-esterine solution. The medulla section of the brain was removed, homogenized and prepared for ultracentrifugation and sucrose density gradient separation. The separated synaptic vesicles were assayed for protein and acetylcholinesterase (ACHE). A decrease in protein was found 2 hours after irradiation with a greater decrease 22 hours later. A decrease in ACHE activity was also seen 2 hours after irradiation but there was no further decrease at 24 hours. These results were correlated with vascular changes found during the central nervous system (CNS) syndrome.</p>			